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AMP-activated protein kinase and hypoxic pulmonary vasoconstriction

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Abstract

Hypoxic pulmonary vasoconstriction is a vital homeostatic mechanism that aids ventilation-perfusion matching in the lung, for which the underlying mechanism(s) remains controversial. However, our most recent investigations strongly suggest that hypoxic pulmonary vasoconstriction is precipitated, at least in part, by the inhibition of mitochondrial oxidative phosphorylation by hypoxia, an increase in the AMP / ATP ratio and consequent activation of AMP-activated protein kinase (AMPK). Unfortunately, these studies lacked the definitive proof that can only be provided by selectively blocking AMPK-dependent signalling cascades. The aim of the present study was, therefore, to determine the effects of the AMPK inhibitor compound C upon: (1) phosphorylation in response to hypoxia of a classical AMPK substrate, acetyl CoA carboxylase, in rat pulmonary arterial smooth muscle and (2) hypoxic pulmonary vasoconstriction in rat isolated intrapulmonary arteries. Acetyl CoA carboxylase phosphorylation was increased approximately 3 fold in the presence of hypoxia ($pO_2 = 16-21$ mm Hg, 1 h) and 5-aminoimidazole-4-carboxamide riboside (AICAR; 1 mM; 4 h) and in a manner that was significantly attenuated by the AMPK antagonist compound C (40 μ M). Most importantly, pre-incubation of intrapulmonary arteries with compound C (40 μ M) inhibited phase II, but not phase I, of hypoxic pulmonary vasoconstriction. Likewise, compound C (40 μ M) inhibited constriction by AICAR (1 mM). The results of the present study are consistent with the activation of AMPK being a key event in the initiation of the contractile response of pulmonary arteries to acute hypoxia.

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Keywords

AMP-activated protein kinase; hypoxic pulmonary vasoconstriction; compound C; AICAR

1. Introduction

Hypoxic pulmonary vasoconstriction is the critical and distinguishing characteristic of the arteries within the lung (von Euler and Liljestrand, 1946), and contributes to ventilation-perfusion matching by diverting blood flow to O₂-rich areas of the lung. The precise mechanism(s) by which hypoxia elicits hypoxic pulmonary vasoconstriction is a contentious issue, although it is clear that relatively mild hypoxia inhibits mitochondrial oxidative phosphorylation in pulmonary arterial smooth muscle (Archer et al., 1986; Duchon and Biscoe, 1992a; b; Leach et al., 2001; Shigemori et al., 1996; Youngson et al., 1993) and over a range of pO_2 that elicits no such response in cells that do not function to monitor O₂ supply (Duchon and Biscoe, 1992b). Despite this fact, however, little consideration has been given to the possibility that hypoxic pulmonary vasoconstriction may be dependent on the subsequent recruitment of AMP-activated protein kinase (AMPK), a central component of a highly conserved protein kinase signalling cascade that monitors the AMP / ATP ratio as an index of metabolic stress (Corton et al., 1995b; Hardie and Hawley, 2001; Marsin et al., 2000; Sakamoto et al., 2005; Shaw et al., 2005; Winder and Hardie, 1996). AMPK is a heterotrimer comprising a catalytic α subunit and regulatory β and γ subunits. It is activated by the binding of AMP to two sites on the γ subunit (Hawley et al., 1995; Scott et al., 2002), leading to allosteric activation of AMPK, phosphorylation of the α subunit at Thr-172 by an upstream kinase, for example the tumour suppressor kinase LKB1 (Hawley et al., 2003; Shaw et al., 2004; Shaw et al., 2005; Woods et al., 2003), and the inhibition of AMPK dephosphorylation. These processes are normally antagonized by high concentrations of ATP, and thereby provide a triple mechanism of regulation that is exquisitely sensitive to very small changes in the AMP / ATP ratio (Hardie and Hawley, 2001). It is generally accepted that AMPK functions to maintain ATP supply in all eukaryotic cells by activating catabolic processes and by inhibiting non-essential ATP consuming processes. However, AMPK is a serine threonine kinase and may regulate processes outside of metabolism (Hardie, 2005). Our most recent investigations suggest that inhibition of mitochondrial oxidative phosphorylation by hypoxia does indeed increase the AMP / ATP ratio in pulmonary arterial smooth muscle, leading to consequent activation of AMPK and, in turn, hypoxic pulmonary vasoconstriction (Evans, 2006; Evans et al., 2006a; Evans et al., 2006b; Evans et al., 2005). However, while these studies provided strong correlations between the effects of AMPK activation on pulmonary artery function and hypoxic pulmonary vasoconstriction, they fell short of demonstrating a causal role for AMPK in the latter process. Here, therefore, we have investigated the effects of the AMPK antagonist compound C on hypoxic pulmonary vasoconstriction.

2. Materials and Methods

2.1 Pulmonary artery isolation and small vessel myography

This investigation conforms to with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996) and all protocols were approved by the University of Georgia Institutional Animal Care and Use Committee. Male Sprague-Dawley rats (250-350g) were killed by decapitation and the heart and lungs rapidly excised and placed in a cold physiological salt solution (PSS) containing (in mM): 118 NaCl, 4 KCl, 1 MgSO₄, 0.435 NaH₂PO₄, 24 NaHCO₃, 1.8 CaCl₂, 5 Na-pyruvate, 5.6 glucose, pH 7.4, 37°C. Small 3rd and 4th order branches of the

pulmonary arterial tree (200-500 μm internal diameter, i.d.) were dissected free of connective tissue and mounted in a small vessel myograph (Model 500A, Danish Myo Technology, Denmark) as previously described in detail (Robertson et al., 2001) and gassed with 95% air / 5% CO_2 . Pulmonary artery rings were then equilibrated with 3 exposures to 80 mM K-PSS (2 min duration, isotonic replacement of Na^+ by K^+), as described previously (Robertson et al., 2001). To facilitate the hypoxic response, pulmonary arteries were exposed to 1 μM prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$) for 30 min prior to, and during, the hypoxic challenge as previously described (Robertson et al., 2000). Hypoxia (16-21 mmHg) was then induced by gassing with 1% O_2 / 95% N_2 / 5% CO_2 for 45 min, after which time the vessels were reoxygenated for 20 min, washed with PSS and subsequently re-exposed to KPSS. Compound C (10-40 μM , a gift from Merck Research Laboratories, Rahway, NJ) was added to the bathing solution 15 min. prior to the induction of hypoxia. Time-matched controls were employed in all experimental protocols. The concentrations of compound C used was selected based upon previous reports where compound C had been found to inhibit AMPK (Lee et al., 2003; Zhou et al., 2001). Likewise, the effect of compound C (40 μM) on constriction induced in response to (1) AMPK activation by 5-aminoimidazole-4-carboxamide riboside (AICAR; 1 mM) and (2) activation of the sarcoplasmic reticulum store-refilling current by the sarco/endoplasmic reticulum Ca^{2+} ATPase inhibitor thapsigargin (1 μM ; without and then with extracellular Ca^{2+} ; (Mathes and Thompson, 1995)).

2.2 Acetyl CoA carboxylase Phosphorylation

All experiments were performed in accordance with the *United Kingdom Animals (Scientific Procedures) Act 1986*. For each sample 32 pulmonary arteries (2nd and 3rd order branches combined) were excised from 8 animals and carefully threaded onto silk surgical thread. They were then placed into an enclosed experimental chamber which was filled with PSS-B: 118 NaCl, 4 KCl, 1 MgSO_4 , 1.2 NaH_2PO_4 , 24 NaHCO_3 , 2 CaCl_2 , 2 MgCl_2 , 5.6 glucose, pH 7.4, 37°C, and bubbled with 75% N_2 , 20% O_2 , 5% CO_2 (normoxia: 150-160 mm Hg) or 93% N_2 , 2% O_2 , 5% CO_2 (hypoxia: 16-21 mm Hg). Artery samples were exposed, in the absence and presence of compound C (40 mM), to either: (1) 2 h of normoxia (2) 1h of normoxia + 1h of hypoxia or (3) 1h of normoxia and 4h in the presence of AICAR (1mM). Tissue samples were then quickly removed from the chamber, placed in 1ml centrifuge tubes and snap frozen in liquid nitrogen. Acetyl CoA carboxylase (ACC) phosphorylation and total ACC protein levels were analysed using pre-cast 3-8% Tris-acetate gels in Tris-acetate buffer. ACC phosphorylation and total ACC protein was measured via dual labelling using phospho-specific antibodies against Ser-221 on ACC2, with secondary anti-sheep antibodies conjugated to IR680 (1 mg ml⁻¹), and streptavidin conjugated to IR800. Fluorescence from the two dyes was measured simultaneously using an Odyssey Infrared Imaging System (Li-Cor Biosciences) (Scott et al., 2002). All tissue samples were assayed in parallel.

2.3 Data presentation and statistical analysis

Contractile responses were calculated as a percentage of the maximal contractile response to KPSS (% T_K) for each vessel. Data for small vessel myography are presented as mean \pm S.E.M., whilst those for ACC phosphorylation represent the mean \pm S.D.. Data were analyzed by repeated measures analysis of variance (ANOVA). Differences between individual means were determined by Student's modified *t*-test using the Bonferroni correction for multiple comparisons between means using the error mean square term from the ANOVA. A value of $P < 0.05$ was deemed to be significant.

2.4 Drugs and Chemicals

5-aminoimidazole-4-carboxamide riboside (AICAR) and compound C were obtained from Molecular Probes. All other drugs and chemicals were obtained from Sigma.

3. Results

3.1 Compound C inhibits ACC phosphorylation in response to hypoxia and AICAR in isolated pulmonary arteries

We first assessed the ability of compound C to inhibit ACC phosphorylation in response to hypoxia and to AMPK activation by AICAR, which is taken up into cells via the adenosine transporter and metabolised to form the AMP mimetic ZMP (Corton et al., 1995a; Owen et al., 2000), in pulmonary arterial smooth muscle. Under normoxia (150-160 mmHg) the ratio of phosphorylated ACC / ACC (pACC/ACC) in 2nd and 3rd order branches of the pulmonary arterial tree, without endothelium, measured 1.00 ± 0.04 in the absence and 0.58 ± 0.10 (mean \pm S.D., $P < 0.05$, $n = 3$) in the presence of 40 μ M compound C. Upon exposure to AICAR (1mM) the pACC/ACC ratio rose to 4.23 ± 0.85 and in a manner that was reversed to 1.49 ± 0.47 ($P < 0.05$; $n = 3$) in the presence of 40 μ M compound C. Likewise, hypoxia (16-21 mmHg) increased the pACC/ACC ratio to 4.12 ± 1.03 in the absence and 2.82 ± 0.63 ($P < 0.05$; $n = 3$) in the presence of 40 μ M compound C. Thus, compound C reduced the resting pACC/ACC ratio and inhibited the increase thereof in response to hypoxia and AMPK activation by AICAR (Fig. 1). These data suggest that compound C is an effective antagonist of AMPK in intact pulmonary arteries.

3.2 Compound C inhibits hypoxic pulmonary vasoconstriction and pulmonary artery constriction in response to AICAR

Consistent with previous reports, in pulmonary artery rings from 3rd and 4th order branches of the pulmonary arterial tree, pre-constricted with 1 μ M PGF_{2 α} , acute hypoxic pulmonary vasoconstriction was biphasic in nature. An initial transient constriction (phase I) peaked at $70.4 \pm 2.4\%$ of the constriction to 80 mM K⁺ (%T_K; mean \pm S.E.M., $n = 10$) and within 5 min. of the onset of hypoxia. This was super-imposed on a slowly developing, sustained constriction (phase II) that peaked at $28.4 \pm 2.4\%$ T_K ($n = 10$) after 40 min of hypoxia. Upon returning to normoxia the vessels rapidly dilated to basal levels of tension (Fig. 2A). When pulmonary artery rings were pre-incubated with compound C (40 μ M, 15 min; Fig. 2B) phase I of hypoxic pulmonary vasoconstriction remained unaffected, measuring $64.82 \pm 1.51\%$ %T_K ($n = 5$). In marked contrast, the magnitude of phase II of hypoxic pulmonary vasoconstriction was markedly reduced to $11.6 \pm 1.4\%$ %T_K ($P < 0.05$; $n = 5$). Fig. 2C shows the mean response to hypoxia for all time points except that immediately following the peak of phase I. In Fig. 2D an exemplar record shows the concentration-dependent inhibition of the sustained phase II of hypoxic pulmonary vasoconstriction by compound C (10-30 μ M; $n = 5$).

Consistent with its effects on hypoxic pulmonary vasoconstriction, compound C inhibited pulmonary artery constriction in response to AMPK activation with AICAR. Thus, in the presence of 1 μ M PGF_{2 α} , exposure of 3rd and 4th order intrapulmonary arteries to AICAR (1mM) resulted in sustained constriction over a 40 minute period (Fig. 3A). Pre-incubation of pulmonary arteries with compound C (40 μ M) significantly inhibited the constriction in response to AICAR (Fig. 3B), which measured $14.6 \pm 1.4\%$ T_K ($n = 5$) and $3.2 \pm 2.3\%$ T_K ($n = 6$) in the absence and presence of compound C, respectively ($P < 0.05$).

3.2 Compound C has no effect on basal tone, and is without effect on pulmonary artery constriction in response to either K-induced depolarisation, PGF_{2 α} or activation of the store-refilling current

Despite the fact that the basal level of ACC phosphorylation in pulmonary arterial smooth muscle was inhibited by compound C, basal tone remained unaffected in the presence of compound C; measuring $13.8 \pm 0.8\%$ %T_K in its absence and $15.2 \pm 1.4\%$ %T_K in the presence of 40 μ M compound C ($n = 5$).

In marked contrast to its effects on hypoxic pulmonary vasoconstriction and constriction in response to AICAR, compound C (40 μ M) had no discernable effect on pulmonary artery constriction in response to 80mM K^+ (i.e. depolarisation and voltage-gated Ca^{2+} influx), which measured $100 \pm 1.0\% T_K$ and $94.4 \pm 3.8\% T_K$, respectively ($n = 5$; Fig. 3AB), or constriction in response to $PGF_{2\alpha}$, which measured $14.7 \pm 1.1\% T_K$ and $13.3 \pm 2.1\% T_K$ in the absence and presence of compound C (40 μ M; $n = 5$), respectively. Furthermore, the constriction in response to Ca^{2+} entry via the store-refilling current following depletion of smooth muscle SR stores with thapsigargin (3 μ M) remained unaffected in the presence of compound C (40 μ M), measuring $44.25 \pm 3.1\% T_K$ in its absence and $47.43 \pm 6.1\% T_K$ its presence (Fig. 4). Note, to deplete SR stores thapsigargin was applied in Ca^{2+} free solution (+ 1mM EGTA; equimolar substitution with Mg^{2+}) and the constriction measured upon subsequent readmission of Ca^{2+} . We may conclude, therefore, that compound C selectively inhibits hypoxic pulmonary vasoconstriction and pulmonary artery constriction in response to AMPK activation by AICAR.

4. Discussion

We have recently shown that physiological levels of hypoxia increase the AMP / ATP ratio in pulmonary artery smooth muscle with concomitant activation of AMPK, and that AMPK activation by AICAR mimics the characteristics of hypoxic pulmonary vasoconstriction (Evans et al., 2006a; Evans et al., 2005). As mentioned previously, however, these studies lacked the definitive evidence that can only be supplied by studying the impact on hypoxic pulmonary vasoconstriction of blocking AMPK-dependent signalling pathways.

In the present investigation we therefore sought to provide such evidence by determining the effects of the AMPK inhibitor, compound C, upon the responses of rat intrapulmonary arteries to acute hypoxia. Consistent with it acting as an effective AMPK inhibitor in intact pulmonary arteries, compound C reduced basal levels of phosphorylated acetyl CoA carboxylase (ACC), a classical AMPK substrate, in pulmonary arterial smooth muscle and partially reversed ACC phosphorylation induced by both hypoxia and AMPK activation in response to AICAR.

Most significantly, however, pre-incubation of isolated pulmonary artery rings with compound C reduced phase II, but not phase I, of hypoxic pulmonary vasoconstriction. In marked contrast, compound C was without effect on constriction in response to the vasoconstrictor $PGF_{2\alpha}$, applied at concentrations which induce pulmonary arterial smooth muscle constriction by mobilising ryanodine-sensitive SR stores via a mechanism distinct from that activated by hypoxia (Dipp and Evans, 2001; Dipp et al., 2001). Furthermore, compound C was ineffective against constriction in response to 80 mM K^+ or Ca^{2+} influx upon activation of the store-refilling current following SR store depletion with thapsigargin.

The specificity of the AMPK inhibitor compound C is not yet well characterized, but AICAR is not known to activate any other protein kinases. Thus, the finding that compound C occludes / reverses hypoxic pulmonary vasoconstriction and constriction by AICAR, but not constriction in response to voltage-gated Ca^{2+} influx, $PGF_{2\alpha}$ or Ca^{2+} influx via the store-refilling current, provides strong support for the view that the effects observed in our study are mediated by inhibition of the AMPK pathway. Our results therefore provide further support for the view that activation of AMPK is *necessary* as well as *sufficient* for hypoxic pulmonary vasoconstriction. However, it should be noted that others have suggested that compound C may inhibit HIF activation by mechanisms independent of AMPK (Emerling et al., 2007).

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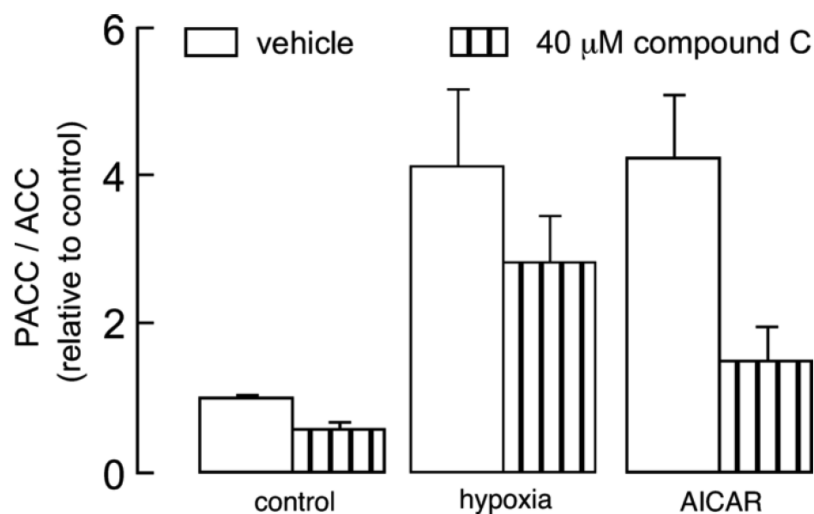


Fig. 1. Phosphorylation of acetyl CoA carboxylase in response to hypoxia and AICAR is inhibited by the AMPK antagonist compound C

Bar chart shows the phosphorylated acetyl CoA carboxylase / acetyl CoA carboxylase (PACC / ACC) ratio measured in pulmonary artery smooth muscle lysates under control conditions (2h normoxia, 150-160 mm Hg), hypoxia (1h at 16-21 mmHg; following 1h normoxia) and in the presence of 1 mM AICAR (4h) with and without 40 μM compound C.

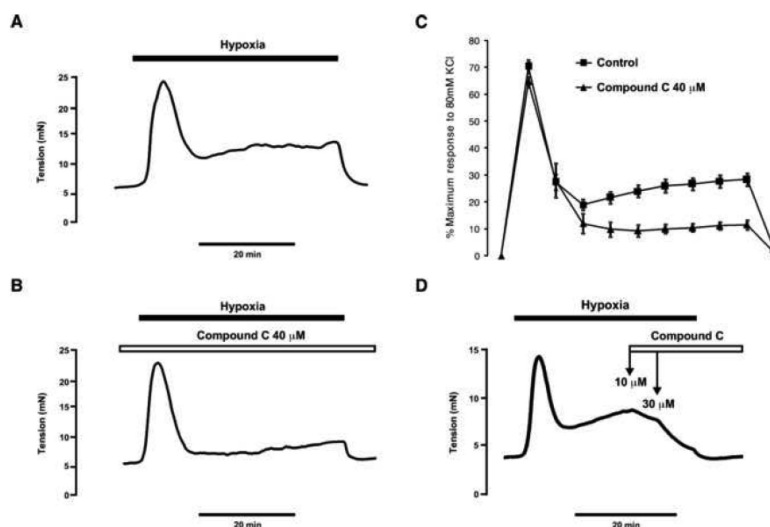


Fig. 2. Hypoxic pulmonary vasoconstriction is concentration-dependently reversed, and inhibited, by Compound C

Panel A shows a typical response of a rat IPA to acute hypoxia that was biphasic in nature, consisting of a transient phase I constriction, superimposed on a sustained phase II contractile response. *Panel B* shows a representative response of a time-matched IPA following pre-incubation (10 min) with compound C (40 μ M). *Panel C* shows the mean effects of pre-incubation with compound C (40 μ M) upon subsequent hypoxic responses in rat IPA ($P < 0.05$ for 40 μ M compound C versus control for all time-points except that immediately following the peak of phase I). *Panel D* shows the concentration-dependent inhibition of the sustained phase II constriction by compound C (10-30 μ M)

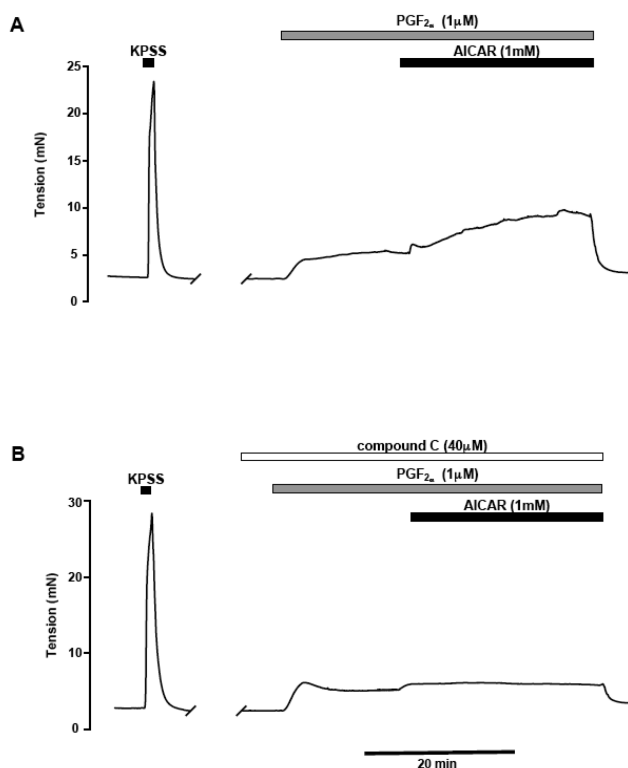


Fig. 3. AICAR-induced pulmonary vasoconstriction is inhibited by Compound C
Panel A shows a typical response of a rat IPA to 1 mM AICAR, which consisted of a slowly-developing sustained constriction. *Panel B* shows the effects of pre-incubation with compound C (40 μM) upon the response to 1 mM AICAR in paired IPA.

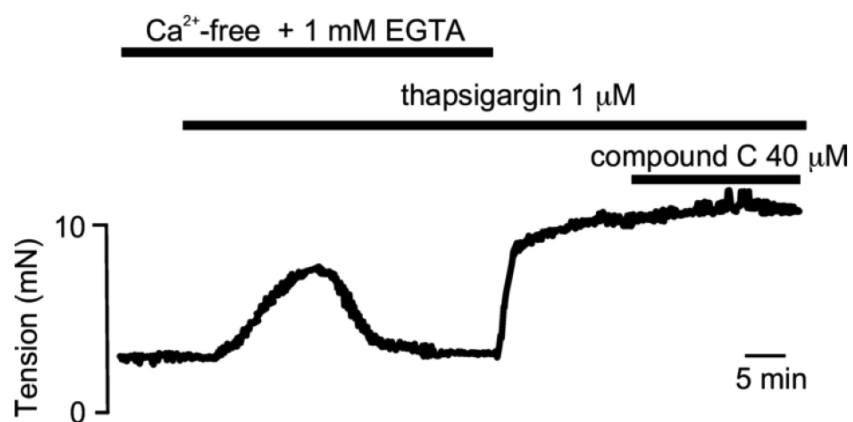


Fig. 4. Thapsigargin-induced pulmonary vasoconstriction is not inhibited by Compound C
Typical response of a rat IPA, without endothelium, to 1 μM thapsigargin in the absence (+ 1 mM EGTA) and then presence of extracellular Ca²⁺ and the effect of subsequent application of compound C (40 μM) to the bathing solution.